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(54) Title: TRANSFORMATION OF *ALLIUM SP.* WITH *AGROBACTERIUM* USING EMBRYOGENIC CALLUS CULTURES

(57) Abstract

The present invention relates to a method for transforming *Allium* species with a heterologous gene using *Agrobacterium.*

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Transformation of *Allium sp.* with Agrobacterium Using Embryogenic Callus Cultures

Technical Field of the Invention

The present invention relates to a method for transforming *Allium* species with a heterologous gene using *Agrobacterium.*

Background of the Invention

Transformation in onion has eluded the scientific community. Initial work on the crop centered around use of biolistics as a means of transforming vegetable monocots (Eady, C.C., Weld, R.J. & Lister, C.E. Transformation of onion, *Allium cepa* L., *Proc. Nat. Onion Research Conference*, Sacramento, CA. USA, Dec. 10-12, 1998). No convincing reports were published showing success using this approach. Recent success was reported in transformation of rice, wheat and corn, using *Agrobacterium* based approaches (U.S. Patent 5,591,616). These reports lead to use of *Agrobacterium* for transformation in monocot vegetables. Recently, Eady (Eady, C.C., Weld, R.J. & Lister, C.E. Transformation of onion, *Allium cepa* L, *Proc. Nat. Onion Research Conference*, Sacramento, CA. USA, Dec. 10-12, 1998) at Crop and Food, NZ, reported on successful transformation of onion using *Agrobacterium* with a kanamycin selectable marker and a Green Florescent Protein (GFP) scoreable marker.

Summary of the Invention

In one embodiment, the present invention relates to a method for transforming an *Allium* species, such as *Allium cepa* or *Allium fistulosum*, with a heterologous gene. Specifically, the method involves contacting embryogenic callus material from an *Allium* species with a bacterium belong to the genus *Agrobacterium* which contains a heterologous gene. The embryogenic callus material is preferably derived from immature embryos or flower buds from an *Allium* species. Preferably, the *Agrobacterium* is *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* and contains a Ti or Ri plasmid. The heterologous gene can be the EPSPS or modified EPSPS gene.

In another embodiment, the present invention further relates to a method for transforming an *Allium* species with a heterologous gene. The first step of the method involves culturing immature embryos or flower buds from an *Allium species* such as *Allium cepa* or *Allium fistulosum* on an initiation medium for a period of from about 2 to about 6 months until embryogenic callus material forms on the embryos or flower buds. Preferably, the immature embryo or flower buds are cultured on the initiation medium in the dark and at a temperature of from about 25°C to about 30°C. The next step of the method involves transferring the embryogenic callus material to a coculture medium and contacting the embryogenic callus material with a suspension of *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* containing a heterologous gene. The next step involves incubating the embryogenic callus with *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* for a period of from about 2 to about 4 days. The next step involves removing the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* from the transformed embryogenic callus material. The final step involves regenerating the transformed embroygenic callus material into transformed *Allium* plants containing the heterologous gene.

Finally, the present invention relates to an *Allium* species transformed by either of the hereinbefore described methods and progeny thereof.

Detailed Description of the Invention

The present invention relates to a method for transforming onion with a heterologous gene using *Agrobacterium* mediated transformation. Any type of onion can be transformed using the method of the present invention, such as, but not limited to *Allium cepa* and *Allium fistulosum*. As used herein, the term "heterologous" when used to describe a gene refers to a gene that originates from a foreign species, or, if from the same species, is substantially modified from its original form. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form.

The method of the present invention employs nodular embroygenic callus material. This embryogenic callus material is preferably derived from immature embryos or from flower buds using techniques which are well known in the art. For example, immature embryos can be obtained from up to fourteen (14) day old post-pollinated flowers. Immature flower buds can be obtained from unopened umbels from an onion.

Once the immature embryos or flower buds are obtained, they are placed on a callus initiation medium such as the initiation medium described in Table A as media number one (#1) and kept under appropriate environmental conditions, specifically, in the dark and at a temperature between from about 25°C to about 30°C, to allow the formation of callus. Other initiation media which induce the formation of callus which are well known in the art, can also be used. For example, any salt formulation media, such as but not limited to, Murshige and Skoog (MS) (Murashige T., Skoog F. (1962) *Physilogia Plantarum* 15:473-497), B-5 (Gamborg, O. L., R. A. Miller, and K. Ojima (1968) "Nutrient requirements of suspension cultures of soybean root cells" *Exp. Cell Res.* 50: 148-151), Heller (Heller, R. (1953) "Recherches sur la nutrition minerale des tissus vegetaux cultivers *in vitro*." *Ann. Sci. Natl. Biol. Veg.* 14: 1 223), White (White. P. R. "Nutrient deficiency studies and an improved inorganic nutrient medium for cultivation of excised tomato roots." *Growth* 7: 53 (1943), which contain a high concentration of auxins (such as indole acetic acid (IAA)), 2,4-diclorophenoxy acetic acid, picloram, indole butyric acid (IBA) as well as a carbon source (such as glucose, sucrose, etc) can be used.

After about two (2) to six (6) months, a nodular embryogenic callus forms on the embryos or flowers. The callus is maintained by subculturing every four (4) weeks, keeping the culture in the dark at a temperature between about 25°C to about 30°C. During this period, any tissue which is not nodular embryogenic callus is removed from the culture. Specifically, the removal of brown or smooth textured tissue and of tissue with anthocyanin or sticky exudates faciliates the development of the nodular

embryogenic callus. The nodular embryogenic callus is the material suitable for transformation with *Agrobacterium*.

For regeneration, the nodular embryogenic callus is transferred to a regeneration medium such as the regeneration medium provided for in Table A as media number two (#2) and is placed under Cool White fluorescent light for about fourteen (14) to about eighteen (18) hours per day at a temperature between about 25°C to about 30°C. Other regeneration media which are well known in the art can also be used. For example, any salt formulation medium, such as, but not limited to, Murshige and Skoog (MS), B-5, Heller, White, which contains low levels of cytokinins (such as benzylaminopurine (BA), kinetin, 6-dimethyallyaminopurine (2IP) and a carbon source (such as glucose, sucrose, etc.) can also be used.

Any desired heterologous or target gene can be introduced into *Allium sp.* using the method of the present invention. The heterologous gene used in the method of the present invention encodes for the expression of a protein, such as the 5-enolpyruvyl-3-phosphate synthase enzyme, which conveys resistance to the glyphosate herbicide. The desired heterologous gene to be inserted into onion can be isolated using molecular biology techniques which are well known in the art or can be produced synthetically using molecular biology techniques which are also well known in the art.

As discussed in the previous paragraph, an example of a heterologous gene that can be used in the method of the present invention is a gene which encodes for the 5-enolpyruvyl-3-phosphate synthase enzyme, which conveys resistance to the glyphosate herbicide. As is well known in the art, glyphosate inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including amino acids, plant hormones and vitamins. Specifically, glyphosate curbs the conversion of phosphoenolpyruvic acid and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvyl-3-phosphate synthase (hereinafter referred to as "EPSPS" or "EPSP synthase"). It is well known that glyphosate-tolerant plants can be produced by inserting into the genome of the plant the

capacity to produce a higher level of EPSP synthase in the chloroplast of the cell which enzyme is preferably glyphosate-tolerant.

Many EPSP synthase genes and the use of these genes to transform plants to make plants which are tolerant to glyphosate herbicides are well known in the art. For example, the nucleotide sequence for the mutant *E. coli* EPSP synthase aroA gene was determined by the method of Sanger, et al. (*Proc. Natl. Acad. Sci. USA* 74:5463) and the corresponding amino acid sequence for the encoded EPSP synthase deduced therefrom. U.S. Patent 4,769,061 discloses a mutated aroA gene which expresses 5-enolpyruvyl-3-phosphoshikimate synthase (EC: 2.5.1.19) (ES-3-P synthase) and methods for making plants which express this mutated gene and which exhibited enhanced resistance to glyphosate herbicides. U.S. Patent 4,940,835 discloses a cloning or expression vector comprising a gene which encodes EPSPS polypeptide which, when expressed in a plant cell contains a chloroplast transit peptide which allows the polypeptide, or an enzymatically active portion thereof, to be transported from the cytoplasm of the plant cell into a chloroplast in the plant cell, and confers a substantial degree of glyphosate resistance upon the plant cell and plants regenerated therefrom. U.S. Patent 5,188,642 discloses how to use the vector described in U.S. Patent 4,940,835 to selectively control weeds in a field. U.S. Patents 5,145,783, 4,791,908 and 5,312,910 describe plant genes, methods for producing said genes and vectors containing these genes which encode a glyphosate-tolerant EPSP synthase where the EPSP synthase has an alanine residue substituted for a glycine residue in a conserved sequence found between positions 80 and 120 in the mature wild-type EPSP synthase. U.S. Patents 5,627,061 and 5,310,667 discloses plant genes encoding EPSP synthases and methods for preparing said genes which are prepared by substituting an alanine residue for a glycine residue in a first conserved sequence found between positions 80 and 120, and either an aspartic acid residue or asparagine residue for a glycine residue in a second conserved sequence found between positions 120 and 160 in the mature wild type EPSP synthase. U.S. Patents 5,633,435 and 5,804,425 disclose a modified EPSPS gene from *Agrobacterium sp.* strain CP4. U.S. Patent 5,866,775 discloses plant genes which encode a glyphosate-tolerant EPSP synthase where the EPSP synthase has an alanine residue substituted for a glycine

residue in a conserved sequence found between positions 80 and 120 and a threonine residue for an alanine residue in a second conserved sequence found between positions 170 and 210 in the mature wild-type EPSP synthase. Additional EPSP synthase genes are disclosed in Padgette et al., *Herbicide Resistant Crops*, Lewis Publisher pages 53-85 (1996). Thereupon, any of the hereinbefore described EPSPS genes can be used in the method of the present invention.

The heterologous gene to be expressed in onion can be used to construct an expression cassette which will be introduced into onion. The construction and composition of expression cassettes is well known in the art. Specifically, the elements of the expression cassette are the heterologous gene, a promoter and a termination DNA segment. The heterologous gene is operatively linked to a promoter DNA segments which controls the expression of the heterologous gene. As used herein, the term "operatively linked"includes reference to a functional linkage between a promoter and the heterologous gene, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the heterologous gene. Generally, operably linked means that the nucleic acid sequences being linked are contigous and, where necessary to joint two protein coding regions, contagious and in the same reading frame. This promoter is not repressed by a product of normal onion metabolism, and can be a constitutive promoter such as the CaMV 35S, octopine synthase promoter (P-Ocs) and nopaline synthase promoter (P-Nos) promoters, or organ-enhanced promoters that cause expression in one or more limited organs of the transformed onion.

The final element in the expression cassette is a termination DNA segment that is operatively linked to the 3' end of the heterologous gene. Several termination segments useful in plants are well known in the art and can be used herein. One exemplary segment is the 3' non-translated region of the nopaline synthase gene (Nos-T). Another is the 3'-non-translated region of the pea rbcS-E9 gene.

In addition, the expression cassette can contain a marker gene which confers a selectable phenotype on the onion cells. For example, the marker may encode biocide

resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to glyphosate or chlorosulforon.

An expression cassette containing the heterologous gene can be introduced into onion using the Ti plasmid of *Agrobacterium tumefaciens* or the Ri plasmid of *Agrobacterium rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by *Agrobacterium* and is stably integrated into the plant genome. Ti and Ri plasmids contain two regions essential for the production of transformed cells. One of these, named transfer DNA (T-DNA), is transferred to plant nuclei and induces tumor or root formation. The other, termed the virulence (vir) region, is essential for the transfer of the T-DNA but is not itself transferred. The T-DNA will be transferred into a plant cell even if the vir region is on a different plasmid. The transferred DNA region can be increased in size by the insertion of heterologous DNA without its ability to be transferred being affected. Thus, a modified Ti or Ri plasmid, in which the disease-causing genes have been deleted, can be used as a vector for the transfer of the gene constructs of this invention into an appropriate plant cell. Construction of recombinant Ti and Ri plasmids in general follows methods typically used to introduce additional DNA into the more common bacterial vectors, such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include, but are not limited to, "shuttle vectors" and structural genes for antibiotic resistance as a selection factor.

The nodular embryogenic callus material prepared as described above is then contacted with the Ti or Ri plasmid of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* which contains the expression cassette with the heterologous gene. After the embryogenic callus material is contacted with the *Agrobacterium*, it is then incubated for about two (2) to about four (4) days at a temperature of about 20°C to about 25°C in the dark. After the incubation period, the *Agrobacterium* is removed or disinfected such as by scraping callus tissue into a dish with wash media, such as the wash medium described in Table B, agitating it and then removing the wash medium.

After removal of the *Agrobacterium*, the washed embryogenic callus material is transferred to a selection medium, such as the selection medium described in Table A as media number four (#4). Other selection media, which are well known in the art, such as media containing the antibiotic kanamycin, can also be used. The callus cultures are grown under Cool White fluorescent light for about 14 to about 18 hours per day at a temperature between about 25°C to about 30°C.

After about thirty (30) days, the callus is subcultured onto a second higher selection media, such as the selection medium described in Table A as media number five (#5), for all following transfers. Selection transfers are done every four (4) weeks per subculture.

Any remaining callus which is living and is producing embryos or plants is then transferred to the rooting media in 0.05 mM glyphosate which is described in Table A as media #6 for final regeneration. Other rooting media which are well known in the art can also be used. The regenerating shoots are grown under Cool White fluorescent light for about 14 to about 18 hours per day at a temperature between about 25°C to about 30°C. Regenerated and rooted shoots are then transplanted into pots filled with soil under high light intensity, such as 1000 foot candles, and at near 100% relative humidity, such as by covering the pots with plastic.

The shoots are allowed to continue to grow and develop into transformed *Allium* plants which contain the heterologous gene. Transformed plants containing the heterologous gene described herein can be identified using techniques known in the art such as Northern or Southern Blotting or polymerase chain reaction.

By way of example and not of limitation, examples of the present invention will now be given.

Example 1: Materials and Methods

a. Callus initiation- Immature embryos from onion, specifically, *Allium cepa* or *Allium fistulosum*, were isolated under a dissecting microscope from approximately 14 day post pollinated flowers. Flower heads can be shipped overnight from various breeding stations around the US, refrigerated and used as explant source for a period of about one (1) to about two (2) weeks. Individual flower buds were removed from the umbel and placed in a 15ml screw cap centrifuge tube. Full strength Clorox plus 0.5% Tween 20 were added to the tube and mixed every 2-3 minutes for 15 minutes. Clorox was removed and buds were washed 4 times with sterile Reverse Osmosis (RO) water. Embryos were isolated by placing the bud on a sterile Petri dish under a 40x dissecting microscope with the flower base facing up. Using a #11 scalpel, the base of the flower was cut to the point of just removing the bottom of the pollinated seed. The seed coat is black and the endosperm is milky to doughy in consistency. The embryos can be squeezed out of the incision on the bottom of the seed with forceps pressure on the top third of the flower bud. However, this procedure may not be successful with older flowers where the endosperm is harder and the embryo is larger. Under these conditions, the seed is extracted from the flower bud for individual embryo excision. These embryos are excised by slicing down the seed coat on the side where the embryo is located. The embryo is extracted from the seed through the incision. Embryos are lifted from the plate on the scapel tip and placed on callus initiation medium (described in Table A as medium #1). Embryos range in size from 1-5 mm.

Plates 60x20mm containing 40ml media can hold up to 25 embryos. A nodular callus forms on the embryo after about 2 to about 4 months. Callus is maintained by subculture for about 3 to about 4 weeks on callus medium #1 shown in Table A. Callus tissue is grown at about 28°C in the dark. Selection of nodular embryogenic tissue is important at each subculture. Removal of brown or smooth consistency tissue, tissue with anthocyanin or sticky exudates promotes development of embryogenic callus.

b. Callus regeneration- Nodular selected tissue is transferred to 60x20mm plates containing 40ml of regeneration medium (described in Table A as medium #2). Cultures are placed under 100 foot candles of Cool White fluorescent light for 16 hours per day at a temperature of about 28° C. Tissue is subcultured at about 3 to about 4 weeks, with embryo regeneration seen at 6-8 weeks.

c. Callus transformation- *Agrobacterium tumefaciens* cultures are initiated from streaked plates of freezer stock. Two loops of plate stock or 100ul of freezer stock are placed in 5ml YEP medium (described in Table B) containing appropriate antibiotics in a 25x150mm tube and placed on a roller drum in room light. Overnight cultures are subcultured by adding 5ml of the overnight culture to 50ml of AB medium (described in Table B) with antibiotics and grown in the dark overnight at 28°C on a gyratory shaker. The next day identified regenerable callus is placed on glass filter paper over co-culture medium (described in Table A as medium #3). Callus tissue is placed on the filter paper at a moderate density. Only nodular tissue is selected for transformation. Overnight *Agrobacterium* cultures are adjusted to an optical density (OD) of from about 0.1-0.4, preferably 0.4, at 660nm with dilution medium (Table B). Diluted cultures are drawn into a plastic sterile transfer pipette. Callus tissue is dabbed with the end of the pipette so a small amount of solution covers the callus tissue. Each callus piece in the plate is touched. The plates are sealed with Parafilm, placed in a black plastic box and incubated at 23°C for 3 days. On day three, *Agrobacterium* is removed by scraping tissue into a 60x20mm plate containing 10ml of wash medium as described in Table B. Tissue is agitated with a transfer pipette followed by removal of the wash. Tissue is scraped into 40ml selection media (described in Table A as medium #4) in a 60x20mm Petri dish and sealed with Parafilm. Cultures are grown under 100 foot candles Cool White florescent light for 16hr/day. After one month, callus is subcultured into a second selection media (described in Table A as medium #5) for 2 transfers and back to selection media #4 (described in Table A) for 1 transfer. Any living callus is transferred to medium #2 (described in Table A) without selection for final regeneration. Regenerating embryos are placed on 50ml rooting medium (described in Table A as medium #6) in Magenta containers and grown under similar light conditions.

Example 2: Specific Experiments

Experiment 212. Callus material used in this experiment was initiated from immature embryos from proprietary *Allium cepa* breeding material owned by Seminis Vegetable Seeds, Inc. Pollinated flowers were sent from Las Cruses, New Mexico to Woodland, California and immature embryos were isolated, using the procedures described in Example 1a from 11 proprietary *Allium cepa* lines. Callus, recently subcultured for seventeen days, from the proprietary *Allium cepa* lines 197,195, 193 and 248 were cocultured on medium #3 (described in Table A) for three days with disarmed Agrobacterium strain ABI containing Monsanto CP4 construct pMON10147 (Monsanto Company, St. Louis, Missouri). The construct pMON10147 contains the enhanced 35S promoter from figwort mosaic virus (which is disclosed in U.S. Patent 5,633,435, hereby incorporated by reference), the leader sequence from the Petunia heat shock protein 70 (HPS70) (disclosed in Winter J., et al., *Mol. Genet.* 211:315-319 (1988), hereby incorporated by reference), the chloroplast transit peptide sequence (CTP2) of the 5-enolpyruvylshikimate-3-phosphate synthase gene (EPSPS) from *Arabidopsis thaliana* which is also disclosed in U.S. Patent 5,633,435, the "modified" EPSPS gene from *Agrobacterium sp.*strain CP4 which is disclosed in U.S. Patent 5,633,435 and the 3' region from the small subunit of ribulose-1,5-bisphosphate gene from *Pisum sativum* (E9) which is also disclosed in Coruzzi, G., et al., *EMBO J.* 3:1671 (1984) and Morelli, G., et al., *Nature*, 315:200-204 (1985), hereby incorporated by reference.

The construct also contains the 35S promoter from cauliflower mosaic virus (CaMV), the chloroplast transit peptide sequence of the small subunit 1a (SSU1a) gene from *Arabidopsis thaliana (*disclosed in Timko M P., Herdies L., Alameida E., Cashmore A R., Leemans J. & Krebbers E. (1988) Genetic engineering of nuclear-encoding components of the photosynthetic apparatus of Arabodopsis. *In* The impact of chemistry on biotechnology – a multidisiplinary discussion- (Phillips M., Schoemaker S.P., Middlekauff D. & Ottenbrite R.M. eds) ACS Books, Washington DC, pp. 279-295), herein incorporated by reference), the modified glyphosate oxidoreductase gene

(GOXsyn) from *Achromobacter sp.* (which is also disclosed in U.S. Patent 5,633,435) and the 3' region of the nopaline synthase gene (nos) from *Agrobacterium tumafaciens* T-DNA.

a. The binary ABI strain contains the disarmed (lacking the T-DNA phytohormones) pTiC58 plasmid pMP9ORK (Koncz, C. and Schell, J., 1986. "The Promoter of TL-DNA Gene 5 Controls the Tissue-Specific Expression of Chimeric Genes Carried by a Novel Type of Agrobacterium Binary Vector," *Mol. Gen. Genet.* 204: 383-396.), in a chloramphenicol resistant derivative of the Agrobacterium tumefaciens strain A208. The pMP9ORK Ti plasmid was engineered to provide the gene functions required for autonomous replication of the plasmid vector after conjugation into the ABI strain. It also provides the vir functions needed for transfer of the T-DNA into the plant cell.

Callus was transferred, after washing, to callus medium #2 (described in Table A) without selection and grown in the dark. Callus was subcultured after 4 weeks on regeneration medium #4 (described in Table A) with 0.1mM glyphosate and moved to the light. Callus was cultured for 3 additional months, with monthly transfers on 0.1mM glyphosate selection (on medium #4 described in Table A) totaling 4 months. Callus line 248 initially established on Gelrite solidified medium (which is medium#1 described in Table A) produced 2 callus lines after glyphosate selection. These lines were subcultured on regeneration medium #2 (described in Table A) without selection. After 2 months , plants were placed on rooting medium #6 (described in Table A).

b. Experiment 268. This experiment employed additional immature embryos obtained from the proprietary line described above in Example 2a. These embryos underwent callus transformation as described above in Example 1c. Moreover, additional callus material used in this experiment was initiated from immature onion flower tissue which originated from proprietary onion line of Seminis Vegetable Seeds, Inc. which is derived from a cross of *Allium fistulosum* x *Allium cepa*. Amphidiploid plant materials of the original *Allium fistulosum* x *Allium cepa* cross (after colchicine-induced chromosome

doubling) was released by Gil McCollum at the U.S.D.A, Beltsville (Notice of Release of Onion Germplasm f-c 8434, 8492, 8497 and 8615, USDA, ARS, Feb. 2, 1988).

To initiate callus from flowers, unopened umbels were cut and sterilized in 20% Clorox for 5 minutes then rinsed with sterile water. Whole flower buds were excised from the umbels and cultured 20 per plate on callus initiation medium #1 (described in Table A). Callus was maintained with monthly subcultures. Eleven flower callus lines were tested for regeneration and found not to regenerate at the frequency of immature embryo derived material. Flower callus line 290011, identified as a regenerating line, was used in experiment 268 along with 16 other embryo derived or flower derived callus lines. Callus was 15 days into its most recent subculture. Callus was cocultured for 3 days with ABI bacteria containing the Monsanto CP4 construct pMON45312 (Monsanto Company, St. Louis, Missouri). Construct pMON45312 contains the enhanced 35S promoter from figwort mosaic virus (FMV) (which is disclosed in U.S. Patent 5,633,435, hereby incorporated by reference), the chloroplast transit peptide sequence (CTP2) of the 5-enolpyruvylshikimate-3-phosphate synthase gene (EPSPS) from *Arabidopsis thaliana* (which is also disclosed in U.S. Patent 5,633,435) , the leader sequence from the soybean heat shock protein (native 17.9) (disclosed in Arfchke, E., et al., *J. Molec. Bio.* 199:549-557 (1988), herein incorporated by reference), the "modified" EPSPS gene from *Agrobacterium* sp. strain CP4 (which is also disclosed in U.S. Patent 5,633,435) , and the 3' region from the small subunit of ribulose-1,5-bisphosphate gene from *Pisum sativum* (E9) which is also disclosed in Coruzzi, G., et al., *EMBO J.* 3:1671 (1984) and Morelli, G., et al., *Nature*, 315:200-204 (1985), hereby incorporated by reference.

The ABI binary *Agrobacterium* strain pTiC58 contains the disarmed (i.e. lacking the T-DNA phytohormone genes) plasmid pMP9ORK (Koncz, C. and Schell, J., 1986. "The Promoter of TL-DNA Gene 5 Controls the Tissue-Specific Expression of Chimeric Genes Carried by a Novel Type of Agrobacterium Binary Vector," *Mol. Gen. Genet.* 204: 383-396), in a chloramphenicol resistant derivative of the *Agrobacterium tumefaciens* strain A208. The pMP9ORK Ti plasmid was engineered to provide the gene functions

required for autonomous replication of the plasmid vector after conjugation into the ABI strain.

Tissue was inducted after washing on regeneration medium #4 (described in Table A) containing 0.05mM glyphosate and grown in the light. After one month, callus was moved to regeneration media #5 (described in Table A) containing 0.1mM glyphosate for 2 transfers. Callus was transferred back to 0.05mM glyphosate regeneration media #4 (described in Table A) for one month. Selected green callus areas were placed on regeneration media #2 (described in Table A) without selection for 2 months. Developing embryos were transferred to elongation rooting medium #6.

Example 3: Discussion

The choice of tissue for transformation in onion or any plant culture system is critical for successful production of transgenic plants. Experiment 212 used immature embryo derived callus of a proprietary *Allium cepa* line. Two selected callus lines which were transformed were regenerated from this experiment aided by the use of a regenerating embryogenic callus line as the initial tissue source.

Immature flowers may also be used as a callus source. Experiment 268 discloses using onion flowers as callus source, however, the initial regeneration screen showed poor regeneration in flower derived callus. The regenerating flower tissue used in Experiment 268 came from a proprietary line which was a *Allium fistulosum* x *Allium cepa* cross that was doubled to become tetraploid. It appeared to be very vigorous in culture and was one of the only flower derived lines that regenerated.

Experiments 212 varies from 268 by selection procedure although both produced transgenic callus lines. Experiment 212 callus was placed on a callus medium without selection and grown the dark. After 1 month, callus was moved to the light and selected on 0.1mM glyphosate for 4 months. Experiment 268 was directly selected on 0.05mM glyphosate on a regenerating medium in the light followed by 2 months selection on

0.1mM glyphosate and a final selection on 0.05mm glyphosate. Experiment 268 produced more lines, however, different genotypes were used.

Delay of selection is used in soybean glyphosate transformation and should be tested further in the onion procedure, however, selection immediately after coculture, as in experiment 268, produced transgenic lines. The reduction of glyphosate selection was done in experiment 268 due to the fact that glyphosate accumulates in tissue and may overwhelm any engineered plant resistance. This is also why regeneration is done without glyphosate selective pressure.

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof. It is intended that all such variations within the scope and spirit of the appended claims be embraced thereby.

Changes can be made to the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention as defined in the following claims.

TABLE A

Onion Media	Callus Regeneration #1	#2	Coculture #3	Selection #4	Selection #5	Rooting #6
MS Salt	4.3 g/l	4.3 g/l	4.3 g/l	4.3 g/l	4.3 g/l	4.3 g/l
B-5 Vitamins	1ml/l	1 ml/l	1 ml/l	1 ml/l	1 ml/l	1 ml/l
Sucrose	30g/l	30 g/l	30g/l	30 g/l	30 g/l	30 g/l
Picloram	1 mg/l					
BA	0.9 mg/l	1 mg/l	1 mg/l	1 mg/l	1 mg/l	
Proline		2.5 g/l	2.5 g/l	2.5 g/l	2.5 g/l	
NaH_2PO_4						170 mg/l
Casein						1 g/l
Kinetin						1 mg/l
Acetosyringone			40 mg/l			
Carbenicillin				500 mg/l	500 mg/l	
Cefotaxime				400 mg/l	400 mg/l	
Glyphosate				0.05mM	0.1mM	0.05mM
Agar // or	7 g/l	7 g/l	7 g/l	7 g/l	7 g/l	6.2 g/l
Phytogel	2.5 g/l					
pH	5.7	5.7	5.7	5.7	5.7	5.8

Table B

YEP Medium

Peptone- 10 g/l
Yeast extract- 10 g/l
NaCl- 5 g/l

AB Medium

Buffer : 20X Final Volume= 500ml
K_2HPO_4. 3H2O- 39.33 g
NaH_2PO_4.H2O- 11.5 g
Filter Sterilize and refrigerate

Salts: 20X Final Volume= 500ml

NH_4Cl-	10g
$MgSO_4.7H_2O$-	12.5g
KCl-	1.5g
$CaCl_2$	0.1g
$FeSO_4$	25mg

Filter Sterilize and refrigerate

Glucose-
50 g/ 500ml

Dilution Medium-

1/10 MSO + 1.0 mg/l BA + 2.5 g/l proline
200uM Acetosyringone
1mM galacturonic acid
20mM MES (2-[N-morpholino]ethanesulfonic acid)
pH 5.4

Wash

MSO **(MS medium plus minimal organics)**
500ug/l Carbenicillin
400 ug/l Cefotaxime

WHAT IS CLAIMED IS:

1. A method for transforming an *Allium* species with a heterologous gene, the method comprising the step of: contacting embryogenic callus material from an *Allium* species with a bacterium belonging to the genus *Agrobacterium* which contains a heterologous gene.

2. The method of claim 1 wherein the *Allium* species is *Allium cepa* or *Allium fistulosum*.

3. The method of claim 1 wherein the bacterium belonging to the genus *Agrobacterium* is *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*.

4. The method of claim 1 wherein the bacterium belonging to the genus *Agrobacterium* contains a Ti plasmid or a Ri plasmid.

5. The method of claim 1 wherein the heterologous gene is the EPSPS gene.

6. The method of claim 5 wherein the heterologous gene is a modified EPSPS gene.

7. The method of claim 1 wherein the embryogenic callus material is derived from immature embryos or flower buds from an *Allium* species.

8. An *Allium* species transformed by the method of claim 1 and progeny thereof.

9. A method for transforming an *Allium* species with a heterologous gene, the method comprising the steps of:

a. culturing immature embryos or flower buds from an *Allium* species on an initiation medium for a period of from about 2 to about 6 months until embryogenic callus material forms on the embryos or flower buds;

b. transferring the embryogenic callus material to a coculture medium and contacting the embryogenic callus material with a suspension of *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* containing a heterologous gene;

c. incubating the embryogenic callus material with the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* for a period of from about 2 to about 4 days; and

d. removing the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* from the transformed embryogenic callus material.

10. The method of claim 9 wherein the *Allium* species is *Allium cepa* or *Allium fistulosum*.

11. The method of claim 9 wherein the immature embryos or flower buds are cultured on the initiation medium in the dark and at a temperature of from about 25°C to about 30°C.

12. The method of claim 9 wherein the heterologous gene is the EPSPS gene.

13. The method of claim 12 wherein the heterologous gene is a modified EPSPS gene.

14. The method of claim 9 further comprising the step of regenerating the transformed embryogenic callus material into transformed *Allium* plants containing the heterologous gene.

15. An *Allium* species transformed by the method of claim 9 and progeny thereof.

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Europäisches Patentamt
Zweigstelle in Den Haag Recherchenabteilung

European Patent Office
Branch at The Hague Search division

Office européen des brevets
Département à La Haye Division de la recherche

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BOHMANN & LOOSEN
08 Mai 2002
Eing.
Frist
Erl.

Datum/Date
06.05.02

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°.
S 10007 EP	00932149.8-1212-US0012463

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Seminis Vegetables Seeds, Inc.

COMMUNICATION

The European Patent Office herewith transmits as an enclosure the European search report for the above-mentioned European patent application.

If applicable, copies of the documents cited in the European search report are attached.

[X] Additional set(s) of copies of the documents cited in the European search report is (are) enclosed as well.

EUROPÄISCHES PATENTAMT - DEN HAAG - EUROPEAN PATENT OFFICE - LA HAYE - OFFICE EUROPÉEN DES BREVETS

REFUND OF THE SEARCH FEE

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

European Patent Office

SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 00 93 2149

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
E	WO 00 44919 A (NZ INST FOR CROP & FOOD RES; EADY COLIN CHARLES (NZ); LISTER CAROL) 3 August 2000 (2000-08-03) * page 5 - page 8; claim 3 * ---	1-8,15	C12N15/82 A01H5/00 //C12N9/10
X Y	WO 99 10512 A (DIRKS ROB ;PEETERS ROGER (NL); NUNHEMS ZADEN BV (NL)) 4 March 1999 (1999-03-04) * example 6 * ---	8,15 1-7,9-14	
E	WO 00 58484 A (UNIV GUELPH ;ROJAS BRENDA (CA); BOWLEY STEPHEN R (CA); DEVEREAUX A) 5 October 2000 (2000-10-05) * page 10, line 27 - page 11, line 8 * ---	1-15	
Y	EADY C C ET AL: "Somatic embryogenesis and plant regeneration from immature embryo cultures of onion (Allium cepa L.)." PLANT CELL REPORTS, vol. 18, no. 1-2, November 1998 (1998-11), pages 111-116, XP002195606 ISSN: 0721-7714 * page 115; figure 1 * ---	1-7,9-14	TECHNICAL FIELDS SEARCHED (Int.Cl.7) A01H C12N C07K
A	EADY C C: "Towards the transformation of onions (Allium cepa)." NEW ZEALAND JOURNAL OF CROP AND HORTICULTURAL SCIENCE, vol. 23, no. 3, 1995, pages 239-250, XP008001672 ISSN: 0114-0671 * the whole document * ---	1-15	
	-/--		

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

4

Place of search	Date of completion of the search	Examiner
THE HAGUE	22 April 2002	Bucka, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C04)

European Patent Office

SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 00 93 2149

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	DONG ET AL: "Agrobacterium-mediated transformation of Javanica rice" MOLECULAR BREEDING: NEW STRATEGIES IN PLANT IMPROVEMENT, KLUWER ACADEMIC PUBLISHERS, NL, vol. 2, no. 3, 1996, pages 267-276, XP002124032 ISSN: 1380-3743 * page 268; figure 1 * ---	1-15	
A	WO 98 37212 A (HALLUIN KATHLEEN D ;PLANT GENETIC SYSTEMS NV (BE)) 27 August 1998 (1998-08-27) * example 1 * ---	1-15	
A	DOMMISSE E M ET AL: "ONION IS A MONOCOTYLEDONOUS HOST FOR AGROBACTERIUM" PLANT SCIENCE (LIMERICK), vol. 69, no. 2, 1990, pages 249-258, XP008001673 ISSN: 0168-9452 * the whole document * -----	1-15	TECHNICAL FIELDS SEARCHED (Int.Cl.7)

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
THE HAGUE	22 April 2002	Bucka, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C04)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 00 93 2149

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

22-04-2002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0044919 A	03-08-2000	AU 1899600 A	18-08-2000
		EP 1144664 A1	17-10-2001
		WO 0044919 A1	03-08-2000
WO 9910512 A	04-03-1999	AU 736349 B2	26-07-2001
		AU 9264798 A	16-03-1999
		CA 2301707 A1	04-03-1999
		CN 1268184 T	27-09-2000
		WO 9910512 A1	04-03-1999
		EP 1009844 A1	21-06-2000
		JP 2001514009 T	11-09-2001
		US 6323396 B1	27-11-2001
WO 0058484 A	05-10-2000	AU 3411400 A	16-10-2000
		WO 0058484 A2	05-10-2000
WO 9837212 A	27-08-1998	AU 727570 B2	14-12-2000
		AU 6002798 A	09-09-1998
		BR 9805900 A	24-08-1999
		CA 2252612 A1	27-08-1998
		EP 0900279 A1	10-03-1999
		WO 9837212 A1	27-08-1998
		JP 2000509612 T	02-08-2000

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US00/12463

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A01H 1/00; C07H 21/04; C07K 14/415; C12N 5/04, 5/14, 9/00, 15/00
US CL :435/419, 252.3, 320.1; 530/370; 536/23.2, 23.6; 800/278, 294, 300

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/419, 252.3, 320.1; 530/370; 536/23.2, 23.6; 800/278, 294, 300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,424,412 A (BROWN et al.) 13 June 1995, see entire document.	1-15
Y	US 5,767,377 A (NAKAJIMA et al.) 16 June 1998, see entire document.	1-15
Y	EADY et al. Transient expression of uidA constructs in in vitro onion (Allium cepa L.) cultures following particle bombardment and Agrobacterium-mediated DNA delivery. Plant Cell Reports. 1996, Vol. 15, No. 12, pages 958-962, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents	
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"&" document member of the same patent family	

Date of the actual completion of the international search 30 JUNE 2000	Date of mailing of the international search report 09 AUG. 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PHUONG BUI Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: LISA V. MUELLER
ROCKEY, MILNAMOW & KATZ, LTD.
TWO PRUDENTIAL PLAZA
180 NORTH STETSON, SUITE 4700
CHICAGO, IL 60601

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference SVS38010310P	Date of Mailing *(day/month/year)* 09 AUG 2000 **FOR FURTHER ACTION** **See paragraphs 1 and 4 below**
International application No. PCT/US00/12463	International filing date *(day/month/year)* 05 MAY 2000
Applicant SEMINIS VEGETABLE SEEDS, INC.	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PHUONG BUI
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/220 (July 1998)★ *(See notes on accompanying sheet)*

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference SVS38010310P	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US00/12463	International filing date *(day/month/year)* 05 MAY 2000	(Earliest) Priority Date *(day/month/year)* 05 MAY 1999
Applicant SEMINIS VEGETABLE SEEDS, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readable form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (See Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: LISA V. MUELLER
ROCKEY, MILNAMOW & KATZ, LTD.
TWO PRUDENTIAL PLAZA
180 NORTH STETSON, SUITE 4700
CHICAGO, IL 60601

RECEIVED
MAY - 4 2001
Rockey, Milnamow & Katz, Ltd.

PCT

WRITTEN OPINION

(PCT Rule 66)

Reply Due 6/27/01

	Date of Mailing *(day/month/year)* 27 APR 2001
Applicant's or agent's file reference SVS38010310P	REPLY DUE within TWO months from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/12463	05 MAY 2000	05 MAY 1999

International Patent Classification (IPC) or both national classification and IPC
Please See Supplemental Sheet.

Applicant
SEMINIS VEGETABLE SEEDS, INC.

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I [X] Basis of the opinion
- II [ ] Priority
- III [ ] Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
- IV [ ] Lack of unity of invention
- V [X] Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI [ ] Certain documents cited
- VII [ ] Certain defects in the international application
- VIII [ ] Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension., see Rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 05 SEPTEMBER 2001

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PHUONG BUI
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/408 (cover sheet) (July 1998)★

WRITTEN OPINION

International application No.
PCT/US00/12463

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-17, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

[X] the claims:
pages 18-19, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

[X] the drawings:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*

WRITTEN OPINION

International application No.
PCT/US00/12463

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	5, 6, 9-15	YES
	Claims	1-4, 7, 8	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-15	NO
Industrial Applicability (IA)	Claims	1-15	YES
	Claims	NONE	NO

2. citations and explanations

Claims 1-4, 7 and 8 lack novelty under PCT Article 33(2) as being anticipated by Eady et al. (Plant Cell Reports, 1996, Vol. 15, p. 958-962). Eady teaches a method for transforming Allium cepa with a heterologous gene by contacting immature embryogenic callus material from Allium cepa with Agrobacterium tumefaciens transformation vector. Agrobacterium tumefaciens inherently possessing a Ti plasmid. Accordingly, Eady anticipated the claimed invention.

Claims 1-15 lack an inventive step under PCT Article 33(3) as being obvious over Eady et al. in view of Brown et al. (US Pat. No. 5,424,412). The teachings of Eady have been discussed above. Eady further teaches transforming Allium cepa by the recited steps set forth in claim 9, the only differences being that Eady teaches monthly subculturing instead of Applicant's specific 2-6 months; and incubating with Agrobacterium tumefaciens for 5 days instead of Applicant's 2-4 days. However, Eady's monthly subculturing is encompassed by Applicant's 2-6 months, since the desired result is the same: formation of callus tissue. Furthermore, there does not appear to be unexpected or surprising results with incubating with Agrobacterium for 2-4 days or 5 days, since the desired result here is also the same: plant tissue transformation by Agrobacterium. 2-4 days or 5 days is routine optimization of experimental parameters absent evidence to the contrary. Thus monthly subculturing and 5 days incubating is, for all intent and purpose, functionally equivalent to Applicant's 2-6 months and 2-4 days, respectively. Eady does not teach transformation with the EPSPS gene. Applicant should note that the "modified EPSPS gene" is considered by the Office to be the same as an unmodified EPSPS gene since Applicant does not indicate how the modified EPSPS gene differs from one which is not modified. Brown teaches expression of a heterologous EPSPS (EPSP synthase) in plants to increase plant tolerance to glyphosate-containing herbicides (col. 6). The plants of Brown include onion (Allium) embryogenic callus material (cols. 7-8). Accordingly, one skilled in the art at the time the invention was made would have been motivated transform the plant of Eady with the EPSPS gene of Brown to express the enzyme necessary to (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): A01H 1/00; C07H 21/04; C07K 14/415; C12N 5/04, 5/14, 9/00, 15/00 and US Cl.: 435/419, 252.3, 320.1; 530/370; 536/23.2, 23.6; 800/278, 294, 300

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
increase plant tolerance to glyphosate-containing herbicides with a reasonable expectation of success.

---------- NEW CITATIONS ----------
NONE

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference SVS88010310P	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/12463	International filing date *(day/month/year)* 05 MAY 2000	Priority date *(day/month/year)* 05 MAY 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant SEMINIS VEGETABLE SEEDS, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04 DECEMBER 2000	16 AUGUST 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PHUONG BUI Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/12463

I. Basis of the report

1. With regard to the **elements** of the international application: *

[X] the international application as originally filed

[X] the description:
pages 1-17 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the claims:
pages 18-19 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the drawings:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELI[redacted]ARY EXAMINATION REPORT

International application No.
P[redacted]S00/12463

V. Reasoned statement under Article 35(2) with regard t novelty, inventive step or industrial applicability; citations and explanations supp rting such statement

1. statement

Novelty (N)	Claims	5, 6, 9-15	YES
	Claims	1-4, 7, 8	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-15	NO
Industrial Applicability (IA)	Claims	1-15	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-4, 7 and 8 lack novelty under PCT Article 33(2) as being anticipated by Eady et al. (Plant Cell Reports, 1996, Vol. 15, p. 958-962). Eady teaches a method for transforming Allium cepa with a heterologous gene by contacting immature embryogenic callus material from Allium cepa with Agrobacterium tumefaciens transformation vector. Agrobacterium tumefaciens inherently possessing a Ti plasmid. Accordingly, Eady anticipated the claimed invention.

Claims 1-15 lack an inventive step under PCT Article 33(3) as being obvious over Eady et al. in view of Brown et al. (US Pat. No. 5,424,412). The teachings of Eady have been discussed above. Eady further teaches transforming Allium cepa by the recited steps set forth in claim 9, the only differences being that Eady teaches monthly subculturing instead of Applicant's specific 2-6 months; and incubating with Agrobacterium tumefaciens for 5 days instead of Applicant's 2-4 days. However, Eady's monthly subculturing is encompassed by Applicant's 2-6 months, since the desired result is the same: formation of callus tissue. Furthermore, there does not appear to be unexpected or surprising results with incubating with Agrobacterium for 2-4 days or 5 days, since the desired result here is also the same: plant tissue transformation by Agrobacterium. 2-4 days or 5 days is routine optimization of experimental parameters absent evidence to the contrary. Thus monthly subculturing and 5 days incubating is, for all intent and purpose, functionally equivalent to Applicant's 2-6 months and 2-4 days, respectively. Eady does not teach transformation with the EPSPS gene. Applicant should note that the "modified EPSPS gene" is considered by the Office to be the same as an unmodified EPSPS gene since Applicant does not indicate how the modified EPSPS gene differs from one which is not modified. Brown teaches expression of a heterologous EPSPS (EPSP synthase) in plants to increase plant tolerance to glyphosate-containing herbicides (col. 6). The plants of Brown include onion (Allium) embryogenic callus material (cols. 7-8). Accordingly, one skilled in the art at the time the invention was made would have been motivated transform the plant of Eady with the EPSPS gene of Brown to express the enzyme necessary to (Continued on Supplemental Sheet.)

Supplemental B x
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII **Sheet 10**

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): A01H 1/00; C07H 21/04; C07K 14/415; C12N 5/04, 5/14, 9/00, 15/00 and US Cl.: 435/419, 252.3, 320.1; 530/370; 536/23.2, 23.6; 800/278, 294, 300

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
increase plant tolerance to glyphosate-containing herbicides with a reasonable expectation of success.

---------- NEW CITATIONS ----------
NONE

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

. or re[redacted] Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* SVS38010310PCT

Box No. I TITLE OF INVENTION

Transformation of *Allium sp.* with Agrobacterium Using Embryogenic Callus Cultures

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Seminis Vegetable Seeds, Inc.
1905 Lirio Avenue
Saticoy, CA 93004

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: United States of America

State *(that is, country)* of residence: United States of America

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Reynolds, John
600 Schmeiser Avenue
Davis, CA 95616

This person is:

☐ applicant only

☐ applicant and inventor

☒ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: United States of America

State *(that is, country)* of residence: United States of America

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Mueller, Lisa V.
Rockey, Milnamow & Katz, Ltd.
Two Prudential Plaza
180 North Stetson, Suite 4700
Chicago, Illinois 60601
U.S.A.

Telephone No.
(312) 616-5400

Facsimile No.
(312) 616-5460

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

1. If, in any of the Boxes, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:

(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;

(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;

(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;

(iv) if, in addition to the agent(s) indicated in Box IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;

(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V., the name of the United States of America is accompanied by an indication "continuation" or "continuation-in-part": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI) , the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

(vi) if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;

(vii) if, in Box No. VI, the earlier application is an ARIPO application: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.

2. If, with regard to the precautionary designation statement contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.

3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty: in such case, write "Statement concerning non-prejudical disclosures or exceptions to lack of novelty" and furnish that statement below.

Continuation of Box IV

Chapa, Lawrence
Elliott, Thomas
Erickson, Randal
Geimer, Steve D.
Hoover, Allen J.
Katz, Martin L.
Lyons, Kathleen A.
Milnamow, John P.
Odell, Paul M.
Polit, Robert B.
Ramesh, Elaine M.
Rockey, Keith V.
Rollins, John
Ross, Thomas I.
Scott, Ted R.
Siegel, Joel
Vargo, Paul V.

Box No. V DESIGNATION OF [redacted]ES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **TZ** United Republic of Tanzania, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ **AE** United Arab Emirates
- ☒ **AL** Albania
- ☒ **AM** Armenia
- ☒ **AT** Austria
- ☒ **AU** Australia
- ☒ **AZ** Azerbaijan
- ☒ **BA** Bosnia and Herzegovina
- ☒ **BB** Barbados
- ☒ **BG** Bulgaria
- ☒ **BR** Brazil
- ☒ **BY** Belarus
- ☒ **CA** Canada
- ☒ **CH and LI** Switzerland and Liechtenstein
- ☒ **CN** China
- ☒ **CR** Costa Rica
- ☒ **CU** Cuba
- ☒ **CZ** Czech Republic
- ☒ **DE** Germany
- ☒ **DK** Denmark
- ☒ **DM** Dominica
- ☒ **EE** Estonia
- ☒ **ES** Spain
- ☒ **FI** Finland
- ☒ **GB** United Kingdom
- ☒ **GD** Grenada
- ☒ **GE** Georgia
- ☒ **GH** Ghana
- ☒ **GM** Gambia
- ☒ **HR** Croatia
- ☒ **HU** Hungary
- ☒ **ID** Indonesia
- ☒ **IL** Israel
- ☒ **IN** India
- ☒ **IS** Iceland
- ☒ **JP** Japan
- ☒ **KE** Kenya
- ☒ **KG** Kyrgyzstan
- ☒ **KP** Democratic People's Republic of Korea
- ☒ **KR** Republic of Korea
- ☒ **KZ** Kazakhstan
- ☒ **LC** Saint Lucia
- ☒ **LK** Sri Lanka
- ☒ **LR** Liberia
- ☒ **LS** Lesotho
- ☒ **LT** Lithuania
- ☒ **LU** Luxembourg
- ☒ **LV** Latvia
- ☒ **MA** Morocco
- ☒ **MD** Republic of Moldova
- ☒ **MG** Madagascar
- ☒ **MK** The former Yugoslav Republic of Macedonia
- ☒ **MN** Mongolia
- ☒ **MW** Malawi
- ☒ **MX** Mexico
- ☒ **NO** Norway
- ☒ **NZ** New Zealand
- ☒ **PL** Poland
- ☒ **PT** Portugal
- ☒ **RO** Romania
- ☒ **RU** Russian Federation
- ☒ **SD** Sudan
- ☒ **SE** Sweden
- ☒ **SG** Singapore
- ☒ **SI** Slovenia
- ☒ **SK** Slovakia
- ☒ **SL** Sierra Leone
- ☒ **TJ** Tajikistan
- ☒ **TM** Turkmenistan
- ☒ **TR** Turkey
- ☒ **TT** Trinidad and Tobago
- ☒ **TZ** United Republic of Tanzania
- ☒ **UA** Ukraine
- ☒ **UG** Uganda
- ☒ **US** United States of America
- ☒ **UZ** Uzbekistan
- ☒ **VN** Viet Nam
- ☒ **YU** Yugoslavia
- ☒ **ZA** South Africa
- ☒ **ZW** Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

- ☐ .....
- ☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 05 MAY 1999	60/132,617	U.S.		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): 1

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA/US

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country (or regional Office)

Box No. VIII CHECK LIST: LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	4
description (excluding) sequence listing part)	:	17
claims	:	2
abstract	:	1
drawings	:	0
sequence listing part of description	:	0
Total number of sheets	:	24

This international application is **accompanied** by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☒ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as items(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify):*

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Mueller, Lisa V., Reg. No. 38,978

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA/
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET
Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference SVS38010310pct

Applicant

Seminis Vegetable Seeds, Inc.

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . 240.00 T

2. SEARCH FEE . . . . . 450.00 S

International search to be carried out by US

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 24 sheets.

first 30 sheets 427.00 b1

_____ x _____ = b2

remaining sheets additional amount

Add amounts entered at b1 and b2 and enter total at B . . . . 427.00 B

Designation Fees

The international application contains 82 designations.

8 x 92.00 = 736.00 D

number of designation fees payable *(maximum 8)* amount of designation fee

Add amounts entered at B and D and enter total at I . . . . . 1,163.00 I

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . 15.00 P

5. TOTAL FEES PAYABLE . . . . . 1,868.00 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☐ authorization to charge deposit account (see below)	☐ bank draft	☐ coupons
☒ cheque	☐ cash	☐ other *(specify)*:
☐ postal money order	☐ revenue stamps	

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ US

☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

04-1644 — Deposit Account No.

05/05/2000 — Date *(day/month/year)*

Signature

Form PCT/RO/101 (Annex) (January 2000)

See Notes to the fee calculation sheet

(19) JAPANESE PATENT OFFICE

PATENT ABSTRACTS OF JAPAN

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(71) Applicant: **WAKUNAGA PHARMACEUT CO LTD**

(72) Inventor: **SUMI SHINICHIRO**
FURUYA HIROAKI

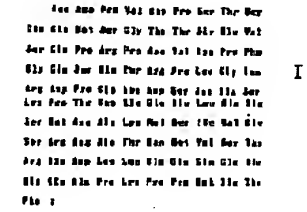
(54) **STRAND-LIKE VIRAL GENE**

(57) Abstract:

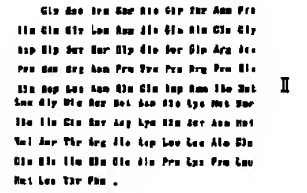
NEW MATERIAL:The title gene having an amino acid sequence of formula I or formula II.

USE: For example, genetic diagnoses for virus infected with garlic.

PREPARATION: Using, as template, virus RNA obtained from purified virus, cDNA is synthesized with oligo(dT) as primer, thus obtaining cDNA clone.



I



II